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PATENT- OG VAREMÆRKESTYRELSEN

Recombinant Poxvirus comprising at least two cowpox ATI promoters

The invention concerns recombinant poxviruses comprising in the viral genome at least two expression cassettes, each comprising the cowpox ATI promoter or a derivative thereof and a coding sequence, wherein the expression of the coding sequence is regulated by said promoter. The virus may be useful as a vaccine or as part of a pharmaceutical composition.

Background of the invention

Recombinant poxviruses are widely used to express foreign antigens in infected cells. Moreover, recombinant poxviruses are currently tested as very promising vaccines to induce an immune response against foreign antigens expressed from the poxvirus vector. Most popular are avipoxviruses on the one side and vaccinia viruses on the other side. US 5,736,368 and US 6,051,410 disclose recombinant vaccinia virus strain Wyeth that expresses HIV antigens and proteins. US 5,747,324 discloses a recombinant Vaccinia virus strain NYCBH expressing lentivirus genes. EP 0 243 029 discloses a recombinant vaccinia virus strain Western Reserve expressing human retrovirus genes. Fowlpoxviruses containing HIV genes in the viral genome are disclosed in US 5,736,368 and US 6,051,410.

To induce an effective immune response it is desirable to express not only a single protein of an agent against which an immune response is to be induced. Instead, it is preferred to express as many different proteins and epitopes of said agent as possible to obtain a broad and effective immunity against said agent. Thus, it might be advantageous to insert several different expression cassettes into the same poxviral genome if it is intended to use a poxvirus as a vector for vaccination. US 5,736,368 describes the construction of a recombinant poxvirus harboring expression cassettes for the HIV-1 env gene and the HIV-1 gag-pol gene. For the expression of the

proteins encoded by the different expression cassettes different promoters were used, namely the vaccinia virus D1 promoter and the 40K promoter. The disadvantage of this strategy is that the activities of the different promoters are not identical resulting in a different level of the proteins expressed from the different expression cassettes.

An almost identical expression level could be obtained if the promoters in the different expression cassettes in the poxvirus genome were identical. However, the disadvantage of this strategy is that there is a risk that undesired recombination events may occur between the homologous/identical promoter sequences. Indeed, it has been shown by Howley et al. (Gene (1996) 172, 233-237) that a recombinant vaccinia virus may be generated that comprises three p7.5 promoters in different locations of the viral genome; however, recombination occurred between the homologous promoter sequences resulting in a mixed genomic population of the recombinant poxvirus. Such a mixed and undefined genomic population that reflects the instability of the viral genome is not acceptable if it is intended to use a recombinant poxvirus for vaccination, in particular for the vaccination of humans.

Object of the invention

It was the object of the present invention to provide stable recombinant poxviruses harboring at least two expression cassettes, preferably for genes that are not naturally part of the poxviral genome, wherein it should be possible to produce the proteins encoded by said at least two different expression cassettes in similar amounts.

Detailed description of the invention

This object has been solved by the provision of recombinant poxviruses comprising in the viral genome at least two expression cassettes, each comprising the cowpox ATI promoter or a derivative thereof and a coding sequence, wherein the expression of the coding sequence is regulated by said promoter.

It was shown by the present inventors that poxviruses comprising two or more copies of the ATI promoter are unexpectedly stably; it was demonstrated that no detectable recombination events occurred between the homologous or even identical ATI promoter sequences. This is in contrast to vaccinia viruses comprising two or more p7.5 promoters in the viral genome.

According to the present invention the poxvirus may be any poxvirus in which the expression of genes should be regulated by the ATI promoter or derivative thereof. Thus, the poxvirus may be any virus of the subfamily of Chordopoxvirinae and Entomopoxvirinae (see Fields Virology 3rd edition, Lippincott-Raven Publishers, Philadelphia, USA, Chapter: 83 , ISBN 0-7817-0253-4). Viruses from the subfamily Chordopoxvirinae are particularly preferred if the recombinant poxvirus is used to express genes in mammalian animals, including humans. Particularly preferred genera

belonging to the subfamily Chordopoxvirinae are Orthopoxviruses, Parapoxviruses, Avipoxviruses, Capripoxviruses, Leporipoxviruses and Suipoxviruses. Most preferred are Orthopoxviruses and Avipoxviruses. Examples for avipoxviruses are canarypoxviruses and fowlpoxviruses. An example for an Orthopoxvirus is vaccinia virus. The vaccinia virus strain that may be used according to the present invention may be any vaccinia virus strain, such as strains Copenhagen, Temple of Heaven, Wyeth, Western Reserve, Elstree, NYCBH and so on. Particularly preferred is Modified Vaccinia Ankara (MVA). MVA has been generated by 516 serial passages on chicken embryo fibroblasts of the Ankara strain of vaccinia virus (CVA) (for review see Mayr, A., *et al.* Infection 3, 6-14 [1975]). As a consequence of these long-term passages the resulting MVA virus deleted about 31 kilobases of its genomic sequence and, therefore, was described as highly host cell restricted to avian cells (Meyer, H. *et al.*, J. Gen. Virol. 72, 1031-1038 [1991]). It was shown, in a variety of animal models that the resulting MVA was significantly avirulent (Mayr, A. & Danner, K. [1978] Dev. Biol. Stand. 41: 225-34). Additionally, this MVA strain has been tested in clinical trials as vaccine to immunize against the human smallpox disease (Mayr *et al.*, Zbl. Bakt. Hyg. I, Abt. Org. B 167, 375-390 [1987], Stickl *et al.*, Dtsch. med. Wschr. 99, 2386-2392 [1974]).

According to the present invention any MVA strain may be used. Examples for MVA virus strains used according to the present invention and deposited in compliance with the requirements of the Budapest Treaty are strains MVA 572 and MVA 575 deposited at the European Collection of Animal Cell Cultures (ECACC), Salisbury (UK) with the deposition numbers ECACC V94012707 and ECACC V00120707, respectively and MVA-BN with the deposition number ECACC V00083008.

The most preferred MVA-strain is MVA-BN or a derivative thereof. The features of MVA-BN, the description of biological assays allowing to evaluate whether a MVA strain is MVA-BN or a derivative thereof and methods

allowing to obtain MVA-BN or a derivative thereof are disclosed in WO 02/42480. The content of this application is included in the present application by reference.

In general terms it is preferred to use viruses that are not harmful for the animal including a human, if the virus is used to vaccinate or to treat the animal including a human. For humans particularly safe poxviruses are the different vaccinia virus strains, such as MVA and avipoxviruses such as fowlpoxvirus and canarypoxvirus.

In order to propagate poxviruses, eukaryotic cells are infected with the virus. The eukaryotic cells are cells that are susceptible to infection with the respective poxvirus and allow replication and production of infectious virus. Such cells are known to the person skilled in the art for every poxvirus species. For MVA an example for this type of cells are chicken embryo fibroblasts (CEF) and BHK cells (Drexler I., Heller K., Wahren B., Erfle V. and Sutter G. "Highly attenuated modified vaccinia Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells" J. Gen. Virol. (1998), 79, 347-352). CEF cells can be cultivated under conditions known to the person skilled in the art. Preferably the CEF cells are cultivated in serum-free medium in stationary flasks or roller bottles. The incubation preferably takes place 48 to 96 hours at 37° C ± 2° C. For the infection MVA is preferably used at a multiplicity of infection (MOI) of 0,05 to 1 $TCID_{50}$ and the incubation preferably takes place 48 to 72 hours at 37 °C ± 2° C.

The sequence of the promoter of the cowpox virus A-type inclusion protein gene (ATI promoter) is known to the person skilled in the art. In this context reference is made to the Genebank entry accession number D00319. A preferred ATI promoter sequence is shown as SEQ ID.: No. 1 and is as follows:

5′ GTTTT GAATA AAATT TTTTT ATAAT AAAT 3′

According to the present invention it is possible to use the ATI promoter as specified in SEQ. ID.:No. 1 or to use a derivative of the ATI promoter, which may be a subsequence of the sequence according to SEQ. ID.:No. 1. The term "subsequence of the sequence according to SEQ. ID.:No. 1" refers to shorter fragments of the sequence of SEQ. ID.:No. 1 that are still active as a promoter, in particular as vaccinia virus late promoter. A typical fragment of the sequence of SEQ. ID.:No. 1 has a length of at least 10 nucleotides, more preferably of at least 15 nucleotides, even more preferably of at least 20 nucleotides, most preferably of at least 25 nucleotides of the sequence of SEQ. ID.:No. 1. The subsequence preferably may comprise nucleotides 25 to 29 of SEQ. ID.:No. 1, i.e. the sequence 5′-TAAAT-3′ located at the 3′ end of SEQ. ID.:No. 1. The subsequence may also comprise nucleotides 22 to 29 of SEQ. ID.:No. 1, i.e. the sequence 5′-TAATAAAT-3′ located at the 3′ end of SEQ. ID.:No. 1.

The promoter may be inserted upstream of a coding sequence in such a way that nucleotides 28 to 29 of SEQ. ID: 1 (underlined in the sequence above) are part of the 5 ′ATG 3′ start codon of translation. Alternatively, the promoter may be separated by several nucleotides from the start codon of translation. The spacer between the 3′ end of the promoter according to SEQ ID.: No 1 and the A in the 5′ ATG 3′ start codon is preferably less than 100 nucleotides, more preferably less than 50 nucleotides and even more preferably less than 25 nucleotides. However, the spacer might even be longer as long as the promoter is still capable of directing the expression of the coding sequence located downstream of the promoter.

The derivative of the ATI promoter can also be a sequence that has one or more nucleotide substitutions, deletions and/or insertions with respect to the sequence of SEQ ID.: No. 1, wherein said derivatives are still active as a promoter, in particular as vaccinia virus late promoter. A sequence having one or more nucleotide substitutions is a sequence in which one or more

nucleotides of the sequence according to SEQ ID.: No. 1 are substituted by different nucleotides. A sequence having one or more nucleotide insertions is a sequence in which one or more nucleotides are inserted at one or more locations of the sequence according to SEQ ID.: No. 1. A sequence having one or more nucleotide deletions is a sequence in which one or more nucleotides of the sequence according to SEQ ID.: No. 1 are deleted at one or more locations. In the derivatives of SEQ ID.: No. 1 deletions, substitutions and insertions may be combined in one sequence.

Preferably the derivative has a homology of at least 40%, more preferably of at least 60%, even more preferably of at least 80%, most preferably of at least 90% when compared to the sequence of SEQ ID.: No.1. According to the most preferred embodiment not more than 6 nucleotides, even more preferably not more than 3 nucleotides are substituted, deleted and/or inserted in the sequence of SEQ ID: No. 1.

In particular, it might be preferable to keep nucleotides 25 to 29 of SEQ. ID.:No. 1, i.e. the sequence 5′-TAAAT-3′ in the promoter to attain maximal promoter activity. It might also be preferable to keep nucleotides 22 to 29 of SEQ. ID.:No. 1, i.e. the sequence 5′-TAATAAAT-3 in the promoter.

A bundle of prior art documents allows the person skilled in the art to predict which derivatives of SEQ ID.: No. 1 still have the biological activity of being active as a poxvirus virus promoter, in particular as a vaccinia virus late promoter. In this context reference is made to Chakrarbarti et al., Biotechniques (1997) 23, 1094-1097 and Davison and Moss, J. Mol. Biol. (1989) 210, 771-784. Moreover, whether a fragment is still active as a poxvirus promoter, in particular a vaccinia virus late promoter can easily be checked by a person skilled in the art. In particular the sequence derivative can be cloned upstream of a reporter gene in a plasmid construct. Said construct may be transfected into a eukaryotic cell or cell line, such as CEF or BHK cells that has been infected with a poxvirus. The poxvirus used for infection is preferably a poxvirus from the same genus and even more preferably the same poxvirus than the poxvirus in which the promoter should

be inserted. The expression of the reporter gene is then determined and compared to the expression of the reporter gene controlled by the promoter according to SEQ ID.: No. 1. A derivative according to the present invention is preferably a derivative having a promoter activity in said test system of at least 10%, preferably at least 30%, more preferably at least 50%, even more preferably at least 70%, most preferably at 90% compared to the activity of the promoter sequence of SEQ ID.: No.1. Also those derivatives of SEQ ID.: No.1 are within the scope of the present invention that have a higher promoter activity than SEQ ID.: No. 1.

According to the present invention the recombinant poxvirus comprises at least two expression cassettes, each comprising an ATI promoter or a derivative thereof. In other words the genome of the recombinant poxvirus may comprise two or more ATI promoters or derivatives thereof. The ATI promoters in the viral genome may be the same or different. Thus, it may be that all of the ATI promoters have the sequence according to SEQ ID.: NO. 1. It may also be that all of the ATI promoters are the same derivative of the sequence according to SEQ ID.: No.1. Alternatively, one or more of the ATI promoters may have the sequence of SEQ ID.: NO. 1 and one or more of the ATI promoters in the same poxviral genome may be derivatives of the sequence according to SEQ ID.: NO. 1. If such a poxviral genome comprises two or more derivatives of the ATI promoter, these derivatives may be the same or different. According to a further alternative all of the ATI promoters in the poxviral genome may be different derivatives of the sequence according to SEQ ID.: NO.1.

In general terms the invention relates to recombinant poxviruses comprising at least two ATI promoters or derivatives thereof in the poxviral genome. Thus, the viral genome may comprise e.g. two, three, four, five, six or more ATI promoters or derivatives thereof in the viral genome.

The ATI promoters or derivatives thereof are usually part of expression cassettes, each comprising a cowpox ATI promoter or derivative thereof and a coding sequence, the expression of which is regulated by said promoters. The coding sequences may be any sequences the expression of which should be controlled by the ATI promoter or derivative thereof.

According to one alternative at least one of the ATI promoters in the poxviral genome may be used to express a gene that is already part of the poxviral genome. Such a gene may be a gene that is naturally part of the viral genome or a foreign gene that has already been inserted into the poxviral genome. In these cases the ATI promoter is inserted upstream of the gene in the poxviral genome, the expression of which is to be controlled by the ATI promoter.

Alternatively or additionally at least one of the ATI promoters or derivatives thereof may be part of an expression cassette that is introduced into the poxviral genome. The expression cassettes comprising an ATI promoter or derivative thereof and a coding sequence may be inserted into any suitable location of the viral genome. Without being bound to the following examples, suitable insertion sites may be selected from: (i) non-essential genes such as the TK-gene, (ii) genes that are necessary for the replication of the virus if the function of said gene is supplemented by the cell that is used for the propagation of the virus; (iii) intergenic regions of the poxviral genome, wherein the term "intergenic region" refers preferably to those parts of the viral genome located between two adjacent genes that comprise neither coding nor regulatory sequences; (iv) naturally occurring deletion sites of the poxviral genome. An example of a virus genome having a naturally occurring deletion site is the genome of MVA, in which certain regions are deleted with respect to the genome of the vaccinia virus strain Copenhagen.

As indicated above, the insertion sites are not restricted to these preferred insertion sites since it is within the scope of the present invention that the expression cassette may be inserted anywhere in the viral genome as long as

it is possible to obtain recombinants that can be amplified and propagated in at least one cell culture system, such as Chicken Embryo Fibroblasts (CEF cells) in the case of MVA and other poxviruses such as vaccinia viruses in general and avipoxviruses.

The different expression cassettes/ATI promoters or derivatives thereof may be inserted into different insertion sites in the poxviral genome.

For various reasons it might be preferable to insert two or more expression cassettes into the same insertion site of the poxvirus genome. However, in such a case it has to be excluded that homologous recombination occurs between the different expression cassettes. Homologous recombination would lead to recombinant viruses in which parts of the expression cassettes are deleted. Since no significant parts of the poxviral vector genome are deleted the resulting recombinants are still viable. Thus, there is no selection for viruses having maintained two or more expression cassettes in the same insertion site. To avoid such undesired recombination events it was state of the art to use different promoters if two or more expression cassettes are inserted into the same insertion site. According to the present invention it is now possible to insert two or more expression cassettes, each comprising an ATI promoter or derivative thereof into the same insertion site since no homologous recombination occurs between the promoters in the expression cassettes.

Thus, according to a preferred embodiment at least two, if not all of the expression cassettes are inserted into the same insertion site in the poxviral genome. In this case the different expression cassettes are directly adjacent with no poxviral sequences between the different expression cassettes or at least with only rather short poxviral sequences between the different expression cassettes.

The methods necessary to construct recombinant poxvirus are known to the person skilled in the art. By way of example, the expression cassette and/or the ATI promoter or derivative thereof may be inserted into the poxviral genome by homologous recombination. To this end a nucleic acid is transfected into a permissive cell line, wherein the nucleic acid comprises the expression cassette and/or the ATI promoter or derivative thereof flanked by nucleotide stretches that are homologous to the region of the poxviral genome in which the expression cassette and/or the ATI promoter or derivative thereof is to be inserted. For MVA permissive cells are CEF cells and BHK cells. The cells are infected with the poxvirus and in the infected cells homologous recombination occurs between the nucleic acid and the viral genome. Alternatively it is also possible to first infect the cells with the poxvirus and then to transfect the nucleic acid into the infected cells. Again recombination occurs in the cells. The recombinant poxvirus is then selected by methods known in the prior art. The construction of recombinant poxviruses is not restricted to this particular method. Instead, any suitable method known to the person skilled in the art may be used to this end.

The ATI promoter in the recombinant poxvirus may be used to control the expression of any coding sequence(s). The coding sequence may preferably code for at least one antigenic epitope or antigen. In this case the recombinant poxvirus may be used to express said antigen after infection of cells in an organism, e.g. a mammalian animal including a human. The presentation of said antigen/epitope may elicit an immune response in the organism that may lead to a vaccination of the organism against the agent from which the antigen/epitope is derived. More specifically the epitope/antigen may by part of a larger amino acid sequence such as a polyepitope, peptide or protein. Examples for such polyepitopes, peptides or proteins may be polyepitopes, peptides or proteins derived from (i) viruses, such as HIV, HTLV, Herpesvirus, Denguevirus, Poliovirus, measles virus, mumps virus, rubella virus, Hepatitis viruses and so on, (ii) bacteria, (iii) fungi.

The proteins, peptides or epitopes expressed from the different expression cassettes may be derived from the same agent, such as a virus, bacteria or fungus. By way of example all products expressed from the expression cassettes may be HIV proteins. If all products are derived from the same agent it is possible to induce a very broad immune response against said agent. Alternatively it is also possible that the proteins, peptides or epitopes expressed from the different expression cassettes are derived from different agents. By way of example, the products derived from the expression cassettes in one poxviral genome are derived from different viruses, such as mumps, measles and rubella virus. According to this embodiment it is possible to use one recombinant poxvirus to induce an immune response against several agents.

Alternatively, at least one of the coding sequences may encode a therapeutic compound such as interleukins, interferons, ribozymes, enzymes and so one.

The recombinant poxvirus according to the present invention may be administered to the animal or human body according to the knowledge of the person skilled in the art. Thus, the recombinant poxvirus according to the present invention may be useful as a medicament (i.e. pharmaceutical composition) or vaccine.

The pharmaceutical composition or the vaccine may generally include one or more pharmaceutical acceptable and/or approved carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers in addition to the recombinant poxvirus. Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycollic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like.

For the preparation of pharmaceutical compositions or vaccines, the recombinant poxvirus is converted into a physiologically acceptable form. This can be done based on the experience in the preparation of poxvirus vaccines used for vaccination against smallpox (as described by Stickl, H. *et al.* [1974] Dtsch. med. Wschr. 99, 2386-2392). For example, if the poxvirus is MVA the purified virus may be stored at -80°C with a titre of $5x10^8$ $TCID_{50}$/ml formulated in about 10mM Tris, 140 mM NaCl pH 7.4. For the preparation of vaccine shots, e.g., 10^1-10^9 particles of the recombinant virus according to the present invention are lyophilized in phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other additives such as antioxidants or inert gas, stabilizers or recombinant proteins (e.g. human serum albumin) suitable for *in vivo* administration. An typical formulation suitable for freeze-drying of recombinant MVA comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l Dextran (MW 36.000 – 40.000), 45 g/l Sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. The glass ampoule is then sealed and can be stored between 4°C and room temperature for several months. However, as long as no need exists the ampoule is stored preferably at temperatures below -20°C.

For vaccination or therapy the lyophilisate can be dissolved in 0.1 to 0.5 ml of an aqueous solution, preferably water, physiological saline or Tris buffer, and administered either systemically or locally, i.e. by parenteral, intramuscular or any other path of administration know to the skilled practitioner. The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner.

Thus, according to a related embodiment the invention relates to a method for affecting, preferably inducing an immunological response in a living animal body including a human comprising administering the virus, the composition or the vaccine according to the present invention to the animal or human to be treated. If the recombinant poxvirus is a recombinant MVA a vaccine shot typically comprises at least 10^2, preferably at least 10^4, more preferably at least 10^6, even more preferably 10^8 to 10^9 $TCID_{50}$ (tissue culture infectious dose) of the virus.

The invention further concerns a method for introducing at least two coding sequences into target cells comprising the infection of the target cells with the virus according to the present invention. The target cell may be a cell in which the virus is able to replicate or a cell that can be infected by the recombinant virus, in which the virus, however, does not replicate, such as all types of human cells in the case of recombinant MVA.

The invention further relates to a method for producing a peptide, protein and/or virus comprising the infection of a host cell with a recombinant virus according to the present invention, followed by the cultivation of the infected host cell under suitable conditions, and further followed by the isolation and/or enrichment of the peptide and/or protein and/or viruses produced by said host cell. If it is intended to produce, i.e. amplify the virus according to the present invention the cell has to be a cell in which the virus is able to replicate such as CEF or BHK cells in the case of recombinant MVA. If it is intended to produce a peptide/protein encoded by the virus, preferably a protein/peptide encoded by a coding sequence, the expression of which is controlled by the ATI promoter or a derivative thereof, the cell may be any cell that can be infected by the recombinant virus and that allows the expression of poxvirus encoded proteins/peptides.

The invention further relates to cells infected with the virus according to the present invention.

Summary of the invention

The invention *inter alia* comprises the following, alone or in combination:

Recombinant poxvirus comprising in the viral genome at least two expression cassettes, each comprising the cowpox ATI promoter or a derivative thereof and a coding sequence, wherein the expression of the coding sequence is regulated by said promoter.

Recombinant poxvirus as above, wherein at least two expression cassettes are inserted into the same insertion site in the poxvirus genome.

Recombinant poxvirus as above, wherein the ATI promoter in at least one of the expression cassettes has the sequence of SEQ ID: No. 1

Recombinant poxvirus as above, wherein the ATI promoter in at least one of the expression cassettes is a derivative of the ATI promoter selected from (i) subsequences of the sequence according to SEQ ID: No. 1 and (ii) sequences having one or more nucleotide substitutions, deletions and/or insertions with respect to the sequence according to SEQ ID: No. 1 or with respect to a subsequence thereof, wherein said subsequences and sequences are still active as promoter in the poxvirus.

Recombinant poxvirus as above, wherein the poxvirus is selected from the group consisting of orthopoxviruses and avipoxviruses.

Recombinant poxvirus as above, wherein the orthopoxvirus is a vaccinia virus and wherein the avipoxvirus is selected from canarypoxvirus and fowlpoxvirus.

Recombinant poxvirus as above, wherein the vaccinia virus is modified vaccinia virus strain Ankara (MVA), in particular MVA-BN and MVA 575,

deposited under numbers V00083008 and V00120707, respectively, at the European Collection of Animal Cell Cultures (ECACC).

Recombinant poxvirus as above, wherein at least one of the expression cassettes is inserted in a naturally occurring deletion site of the MVA genome with respect to the genome of the vaccinia virus strain Copenhagen.

Recombinant poxvirus as above, wherein at least one of the expression cassettes is inserted in an intergenic region of the poxvirus genome.

Recombinant poxvirus as above, wherein at least one of the coding sequences codes for least one antigen, antigenic epitope, and/or a therapeutic compound.

Recombinant poxvirus as above as vaccine or medicament.

Vaccine or pharmaceutical composition comprising a recombinant poxvirus as defined above.

Use of the recombinant poxvirus as defined above for the preparation of a vaccine or medicament.

Method for introducing coding sequences into target cells comprising the infection of the target cells with the virus as defined above.

Method for producing a peptide, protein and/or virus comprising (a) infection of a host cell with the recombinant poxvirus according to anyone of claims 1 to 10, (b) cultivation of the infected host cell under suitable conditions, and (c) isolation and/or enrichment of the peptide and/or protein and/or viruses produced by said host cell.

Method for affecting, preferably inducing an immunological response in a living animal body including a human comprising administering the virus as defined above or the composition or vaccine as defined above to the animal or human to be treated.

Method as above comprising the administration of at least 10^2 $TCID_{50}$ (tissue culture infectious dose) of the virus.

A cell containing the virus as defined above.

A method for the production of a recombinant virus as defined above comprising the step of inserting at least two expression cassettes into the genome of a poxvirus.

Short Description of the Figures

Figure 1 and figure 2: Schematic presentation of the recombination vectors pBN70 (figure 1) and pBN71 (figure 2)

F1A137L = Flank 1 of region of insertion; F2A137L = Flank 2 of region of insertion; F2rpt = repeat of flank2; prATI = ATI promoter; pr7.5 = p7.5 promoter; GUS = GUS coding region; NS1 = NS1 coding region; NPTII = Neomycin resistance; IRES = internal ribosomal entry site; EGFP = enhanced green fluorescence protein coding region; AmpR = Ampicillin resistance gene.

Example

The following example will further illustrate the present invention. It will be well understood by a person skilled in the art that the provided example in no way may be interpreted in a way that limits the applicability of the technology provided by the present invention to this example.

Stable Insertion of two foreign genes regulated by the Cowpox ATI promoter in a single site of the MVA genome

The aim of this example was to demonstrate that an insertion of two foreign genes both regulated by the ATI promoter is stable.

Introduction:

The cowpox ATI promoter was fused to the GUS gene (E. coli ß-Glucuronidase) and non-structural (NS) 1 gene of Dengue virus, respectively. For comparison the GUS gene was also fused to the naturally occurring Vacciniavirus pr7.5 promoter. The ATI promoter-NS1 gene expression cassette and either the ATI promoter-GUS gene expression cassette or the p7.5 promoter-GUS gene expression cassette were inserted into a recombination vector comprising sequences homologous to the MVA genome. (Fig. 1 and 2). In the resulting plasmids pBN70 (ATI promoter-NS1 gene expression cassette and ATI promoter-GUS gene expression cassette) and pBN71 (ATI promoter-NS1 gene expression cassette and p7.5 promoter-GUS gene expression cassette) the expression cassettes were flanked by sequences homologous to the sequences in the MVA genome in which the expression cassette was to be inserted. CEF cells were infected with MVA-BN and transfected with pBN70 and pBN71, respectively. In the cells homologous recombination occurred between the MVA genome and the recombination plasmid resulting in a recombinant MVA genome. After several rounds of purification the virus was passaged 20 times and it was

analyzed by sequencing whether the inserted expression cassette was still intact.

Materials and Equipment:

primary CEF cells; MVA-BN with a titre of 10^8 $TCID_{50}$/ml; Effectene transfection kit (Qiagen); VP-SFM cell culture media (Gibco BRL); G418 (Gibco BRL); DNA Nucleospin Blood Quick Pure Kit (Macherey Nagel); Expand high fidelity DNA polymerase (Roche); Oligos (MWG); Sequencing DCTS Quickstart Kit (Beckman Coulter).

Method

The recombination vectors pBN70 and pBN71 (Fig. 1 and 2) were cloned according to standard protocols known by persons skilled to the art.

5×10^5 CEF cells were seeded per transfection reaction in a well of a 6-well-plate and maintained in VP-SFM over night at 37°C and 5% CO_2. The cells were infected with MVA-BN (moi 1.0) in 0.5 ml VP-SFM per well and incubated for 1 h at room temperature on a shaker. Transfection of linearized pBN70 and 71 was performed as described in the manufacturer protocol (Qiagen).

The resulting recombinant viruses were passaged several times under selective conditions (G418, 300 µg/ml) and single plaques were isolated, amplified and analysed until purified clones were generated. The analysed virus finally was passaged 20 times.

The inserted genes of the purified clones of and the flanking regions were amplified by PCR and sequenced.

Results

The recombination plasmids pBN70 and pBN71 were analyzed by restriction analysis and the sequence of these plasmids was confirmed by sequencing.

The recombinant virus resulting from the recombination of MVA-BN with pBN70 was termed mBN30. The recombinant virus resulting from the recombination of MVA-BN with pBN71 was termed mBN31. Both viruses were plaque purified. After 20 passages in CEF cells viral DNA was isolated. The region, which contains the two foreign genes, the promoters and the flanking regions of the insertion site (Fig. 1, F1A137L up to F2 rpt) was amplified by PCR with a proof reading DNA polymerase and sequenced.

The sequence was shown to be as expected for both viruses. Thus, it was demonstrated that the insertion of two foreign genes regulated by the cowpox ATI promoter is stable.

Claims:

1. Recombinant poxvirus comprising in the viral genome at least two expression cassettes, each comprising the cowpox ATI promoter or a derivative thereof and a coding sequence, wherein the expression of the coding sequence is regulated by said promoter.

2. Recombinant poxvirus according to claim 1, wherein at least two expression cassettes are inserted into the same insertion site in the poxvirus genome.

3. Recombinant poxvirus according to anyone of claims 1 to 2, wherein the ATI promoter in at least one of the expression cassettes has the sequence of SEQ ID: No. 1

4. Recombinant poxvirus according to anyone of claims 1 to 2, wherein the ATI promoter in at least one of the expression cassettes is a derivative of the ATI promoter selected from
   (i) subsequences of the sequence according to SEQ ID: No. 1
   (ii) sequences having one or more nucleotide substitutions, deletions and/or insertions with respect to the sequence according to SEQ ID: No. 1 or with respect to a subsequence thereof,

   wherein said subsequences and sequences are still active as promoter in the poxvirus.

5. Recombinant poxvirus according to anyone of claims 1 to 4, wherein the poxvirus is selected from the group consisting of orthopoxviruses and avipoxviruses.

6. Recombinant poxvirus according to claim 5, wherein the orthopoxvirus is a vaccinia virus and wherein the avipoxvirus is selected from canarypoxvirus and fowlpoxvirus.

7. Recombinant poxvirus according to claim 6, wherein the vaccinia virus is modified vaccinia virus strain Ankara (MVA), in particular MVA-BN and MVA 575, deposited under numbers V00083008 and V00120707, respectively, at the European Collection of Animal Cell Cultures (ECACC).

8. Recombinant poxvirus according to anyone of claims 1 to 7, wherein at least one of the coding sequences codes for least one antigen, antigenic epitope, and/or a therapeutic compound.

9. Recombinant poxvirus according to anyone of claims 1 to 8 as vaccine or medicament.

10. Use of the recombinant poxvirus according to anyone of claims 1 to 8 for the preparation of a vaccine or medicament.

Abstract

The invention concerns recombinant poxviruses comprising in the viral genome at least two expression cassettes, each comprising the cowpox ATI promoter or a derivative thereof and a coding sequence, wherein the expression of the coding sequence is regulated by said promoter. The virus may be useful as a vaccine or as part of a pharmaceutical composition.

Figure 1:

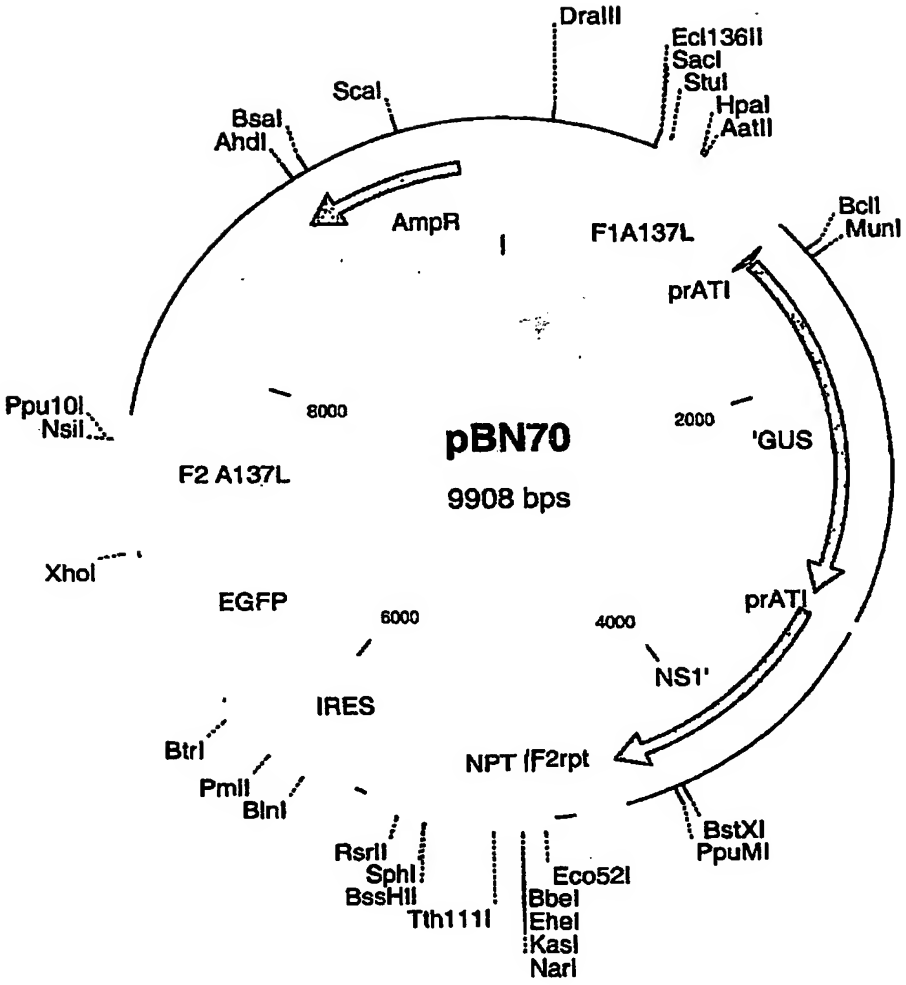
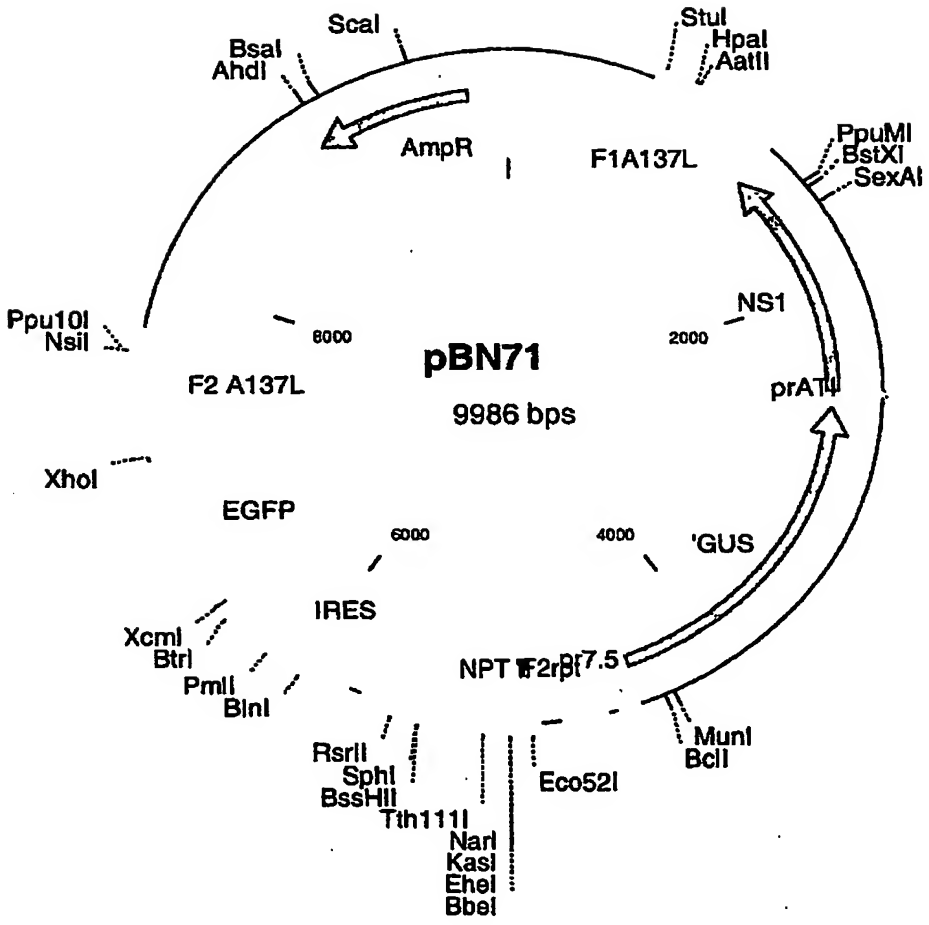


Figure 2:



SEQUENCE LISTING

<110> Bavarian Nordic A/S

<120> Recombinant Poxvirus comprising at least two cowpox ATI promoters

<130> BN52DK

<160> 1

<170> PatentIn version 3.1

<210> 1
<211> 29
<212> DNA
<213> Cowpox virus

<220>
<221> promoter
<222> (1)..(29)
<223>

<400> 1
gttttgaata aaatttttt ataataaat 29

IN 52 UK

Modtaget PVS
25 NOV. 2002

Assignment

We the undersigned

Bavarian Nordic GmbH, Fraunhoferstr. 18b, D-82152 Martinsried, Germany

owner of the rights concerning the invention

„Recombinant Poxvirus Comprising at least two Cowpox ATI Promoters"

herewith assign the rights to

Bavarian Nordic A/S, Ved Amagerbanen, DK-2300 Copenhagen S, Denmark.

21.11.02
Date

Petra Pielken
Petra Pielken
- Patent Director -

We hereby agree to the afore-mentioned assignment

22/11/02
Date

Peter Wulff
- CEO -

Patent- og Varemaerkestyrelsen
Helgeshoej Allé 81
DK-2630 Taastrup

Denmark

Modtaget PVS
25 NOV. 2002

General Authorization

The undersigned

Bavarian Nordic A/S
Vesterbrogade 149
DK-1620 Copenhagen V

Denmark

does hereby authorize

Dr. Petra Pielken
Bavarian Nordic GmbH
Fraunhoferstr. 18b
D-82152 Martinsried

Germany

to act on behalf of Bavarian Nordic A/S in all matters concerning danish patent applications and patents, including withdrawal of danish applications and patents.

This authorization remains in force until it is replaced by another authorization or revoked in writing to the Danish Patent and Trademark Office.

Place: Copenhagen
Date: 01 July 2002

Signature:

Bo Sandroos
Vice President of Bavarian Nordic A/S

BAVARIAN NORDIC

Bavarian Nordic A/S
Vesterbrogade 149, DK-1620 Copenhagen V, Denmark, Phone +45 33 26 83 83, Fax +45 33 26 83 80
www.bavarian-nordic.com, A/S Reg. No. 208 618, VAT No. DK 16 27 11 87

Modtaget PVS

25 NOV. 2002

Patent- og Varemærkestyrelsen
Erhvervsministeriet

Helgeshøj Allé 81
2630 Taastrup

Tlf. 43 50 80 00
Fax 43 50 80 01
Postgiro 8 989 923
E-post pvs@dkpto.dk
www.dkpto.dk

Oplysning om deponering af biologisk materiale

Ansøgningen omfatter følgende deponeringer i henhold til Patentlovens § 8a, stk. 1 eller Brugsmodellovens § 8, stk. 1:

A. Identifikation af deponeringer

1 Vedrørende det på side 4 linie 24-28 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
ECACC
European Collection of Cell Cultures

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Centre for Applied Microbiology & Research
Salisbury
Wiltshire SP4 OJG, United Kingdom

Dato for deponering 7 Dec 2000 Løbenummer 00120707

2 Vedrørende det på side 4 linie 24-28 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
ECACC
European Collection of Cell Cultures

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Centre for Applied Microbiology & Research
Salisbury
Wiltshire SP4 OJG, United Kingdom

Dato for deponering 30 Aug 2000 Løbenummer 00083008

3 Vedrørende det på side 4 linie 24-28 i beskrivelsen omtalte biologiske materiale

Deponeringsinstitutionens navn
ECACC, CAMR
European Collection of Cell Cultures

Deponeringsinstitutionens adresse (inklusive postnummer og land)
Centre for Applied Microbiology & Research, CAMR
Porton Down
Salisbury, SP4 OJG, United Kingdom

Dato for deponering 27Jan 1994 Løbenummer 94012707

☐ Yderligere oplysninger på et følgende ark

B. Yderligere angivelser, fx om det biologiske materiales farlighed, geografisk oprindelse.

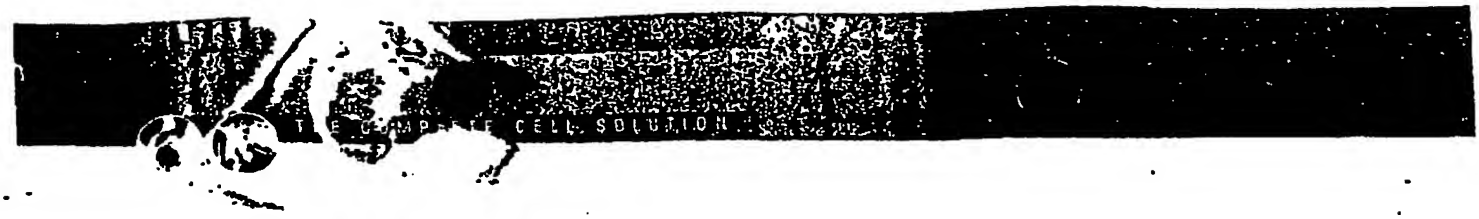
☑ Oplysningerne fortsættes på et vedføjet ark

Vedlagt for begge stammer "Certificate of deposit", "Certificate of Analysis", "Patent deposit Accession Form - Virus" og "Biohazard statement" fra ECACC

C ☑ Det begæres, at udlevering af en prøve, i tiden indtil ansøgningen er fremlagt eller endeligt afgjort uden at være fremlagt, kun sker til særlig sagkyndig, jfr PL § 22, stk 7 eller BML § 8, stk 2

Dato og underskrift 21 November 2002 Petra Pielken.

17 mar00/ls



Modtaget PVS
25 NOV. 2002

ecacc

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref. V00120707) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 7TH December 2000

.....

Dr P J Packer
Quality Manager, ECACC

European Collection of Cell Cultures. CAMR, Salisbury, Wiltshire SP4 0JG UK
Tel: 44 (0) 1980 612512 Fax: 44 (0) 1980 611315 Email. ecacc@camr.org.uk Web Site ecacc.org.uk

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
..VA-575	V00120707

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 7TH December 2000 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV. INTERNATIONAL DEPOSITORY AUTHORITY

Name: Dr P J Packer

Address: ECACC
CAMR
Porton Down
Salisbury SP4 OJG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s):

Date:

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY OF STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: BAVARIAN NORDIC RESEARCH INSTITUTE GMBH Address: FRAUNHOFERSTRASSE 18B D-82152 MARTINSRIED GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: 00120707 Date of the deposit or of the transfer: 7TH December 2000

II. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [1]. On that date, the said microorganism was

[ ] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2 (a) (ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]	
MVA-575 - V00120707 THIS VIRUS WAS TITRATED ON BHK CELLS $TCID_{50} = 10^{6.8}$	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Dr P J Packer ECACC CAMR Address: Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 23/3/01

4 Fill in if the information has been requested and if the results of the test were negative.

Certificate of Analysis

Product Description MVA-575
Accession Number 00120707

Test Description: Determination of $TCID_{50}$ of cytopathic Virus titration. (SOP ECACC/055) Cell

Acceptance Criterion/Specification/Criteria: Negative controls should show no sign of Cytopathic effects. The Test Sample is serially diluted into in 4 wells of indicator cell lines for each dilution. Cytopathic effects indicate that virus is present. Virus titre is calculated using the below equation where x is the value obtained from a standard $TCID_{50}$ Table as a result of the distribution of the wells displaying less than 4 positive wells per dilution, and y is the value of the highest dilution where all 4 wells are positive:

$$TCID_{50} = \frac{1}{y} \times 10^{1+x}$$

Date: 19/01/01

Result:

Indicator Cell Line:	BHK 21 CLONE 13
Negative Control:	NO CPE
Test Sample:	CPE
Distribution of less that 4 positive wells:	4, 4, 0
X:	0.50
Y:	10^{-5}

$$TCID_{50} = \frac{1}{10^{-5}} \times 10^{1+0.50}$$

$$= 10^{6.5}$$

Overall Result: Virus Present

Test Description: The Detection of Mycoplasma by Isolation on Mycoplasma Pig Serum Agar and in Mycoplasma Horse Serum Broth.
SOP QC/MYCO/01/02

Acceptance Criterion/Specification: All positive controls (*M. pneumoniae & M. orale*) must show evidence of mycoplasma by typical colony formation on agar plates. Broths are subcultured onto Mycoplasma Pig Serum Agar where evidence of mycoplasma by typical colony formation is evaluated. All negative control agar plates must show no evidence of microbial growth.
The criteria for a positive test result is evidence of mycoplasma by typical colony formation on agar. A negative result will show no such evidence.

Test Number: 21702
Date: 12/02/01
Result:

Positive Control:	Positive
Negative Control:	Negative
Test Result:	Negative
Overall Result:	PASS

Authorised by.....SJL.....**ECACC, Head of Quality**...5/2/01..... **Date**

Certificate of Analysis

Product Description MVA-575
Accession Number 00120707

Test Description: Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system.
SOP QC/MYCO/07/05

Acceptance Criterion/Specification: The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence. Positive control (*M. orale*) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA. Positive test results appear as extra nuclear fluorescence of mycoplasma DNA. Negative results show no cytoplasmic fluorescence.

Test Number: 21702

Date: 12/02/01

Result:

Positive Control:	Positive
Negative Control:	Negative
Test Result:	Negative
Overall Result:	PASS

Test Description: Detection of bacteria and fungi by isolation on Tryptone Soya Broth (TSB) and in Fluid Thioglycollate Medium (FTGM). SOP QC/BF/01/02

Acceptance Criterion/Specification: All positive controls (*Bacillis subtilus, Clostridium sporogenes* and *Candida albicans*) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear).
The criteria for a positive test is turbidity in any of the test broths. All broths should be clear for negative test result.

Test Number: 21702

Date: 12/02/01

Result:

Positive Control:	Positive
Negative Control:	Negative
Test Result:	Negative
Overall Result:	PASS

Authorised by.....**ECACC, Head of Quality**..... **Date**

ecacc
European Collection of Cell Cultures

ECACC use only
Accession No:
Depositors Code:

Patent Deposit Accession Form - Virus

DEPOSITOR INFORMATION

Name of Depositor/Company/Institute: Bavarian Nordic Research Institute GmbH

(NB this will be the name that appears on certification)

Contact Name: Dr. Paul Howley, Dr. Petra Pielken

Depositor Address: Fraunhoferstraße 18b, D-82152 Martinsried, Germany

Tel No: 89 8565 0030 Fax No: ++49 89 8565 1333

BIOHAZARD STATEMENT MUST BE ENCLOSED

The deposit is made in accordance with the terms of the Budapest Treaty 1977. I agree to abide by the conditions and regulations regarding deposit of cell lines to the ECACC.

Signature: P. Pielken Date: 05.12.2000

Address to which invoice should be sent (if different from above)

Accounts Department, Bavarian Nordic Research Institute GmbH
Fraunhoferstraße 18b
D-82152 Martinsried, Germany

VIRUS INFORMATION

Name in full: Modified Vaccinia Virus Ankara

Abbreviated Name: MVA Identification on Ampoules:

Strain: No. 575 Serological Type:

Normal Host: None

Virus Titre Deposited:

VIRUS PROPAGATION

Host cells (first choice): Chicken Embryo Fibroblast (CEF)

Alternative Host Cells:

Details of Host Cell Growth (media, temperature, seeding density, growth factors etc)

Chicken Embryo Fibroblast Cultured in RPMI Media Supplemented with 10% FCS.
At 37°C/5%CO2. No Growth Factors Needed.

Details of Virus Growth (eg confluency of host cells, co-cultivation, moi, effects, time taken)

Infect CEF Cell At Near Cell Confluency (Approx. 90%) At MOI 0.1 TCID50/Cell

VIRUS STORAGE Confluency; Infection Times on Average 3 Days At 37°C/5%CO2

Material stored (eg supernatant, infected cell extract, viable infected cells etc)

Temperature and conditions: Infected Cell Extract, At-80°C

VIRUS ASSAY

Method (enclose if necessary)

Does not form Plaques. It forms Foci of CPE in CEF Monolayers. Titrate by TCID50 Method - Reference:

LITERATURE REFERENCES (if any) Ingo Drexler et al. 2000 in Methods in Molecular Medicine Vol 35; Gene Therapy: Methods and Protocol s. Ed. W. Walther and U. Stein. Human Press

ANY OTHER RELEVANT INFORMATION Virus Looses Viability At Low ph. Dilute Virus With Sterile IMM Tris-Hcl pH9 Buffer

European Collection of Cell Cultures, Centre for Applied Microbiology & Research
Salisbury, Wiltshire SP4 0JG, UK.
Tel: +44 1980 612512 *Fax:* +44 1980 611315
E.Mail: ecacc@camr.org.uk *Web Site:* www.camr.org.uk

CAMR
Today's Research Tomorrow's Health

REGISTERED FSS

No. FS31819

ecacc
European Collection of Cell Cultures

ECACC use only
Accession No:
Depositors Code:

BIOHAZARD STATEMENT

(To be included with all deposits)

Deposit category

Cell Culture ☐ Plant Culture ☐ Virus Recombinant DNA ☐ DNA Probe ☐ Bacteria ☐

Does the above deposit represent an infectious, toxic or allergenic hazard? Yes ☐ No ☒

If yes, please give details and any associated hazard category (eg. ACDP category) and fax to ECACC PRIOR to shipment of cells.

MVA is classified into biosafety level 1 (S1) by ZKBS
File No.: 6790-10-14
Date: May 1997

Does the above deposit contain genetically manipulated material? Yes ☐ No ☒

If yes, please enclose a general description and answer the following:

a. is the material DNA ☐ RNA ☐

b. is the material present in a host organism? Yes ☐ No ☐

c. is the genetic material readily transferred to environmental organisms? Yes ☐ No ☐

d. is the genetic material likely to be expressed as protein? Yes ☐ No ☐

e. what is the category of this material under ACGM regulations?

ie, i. containment level _____

ii. GMO type _____

For any positive responses to questions b-d please give details

Please supply any further details which would be relevant to assessing the safe handling conditions for materials to be deposited at ECACC.

Signed _____ Date _____

Print name Dr. Petra Pielken

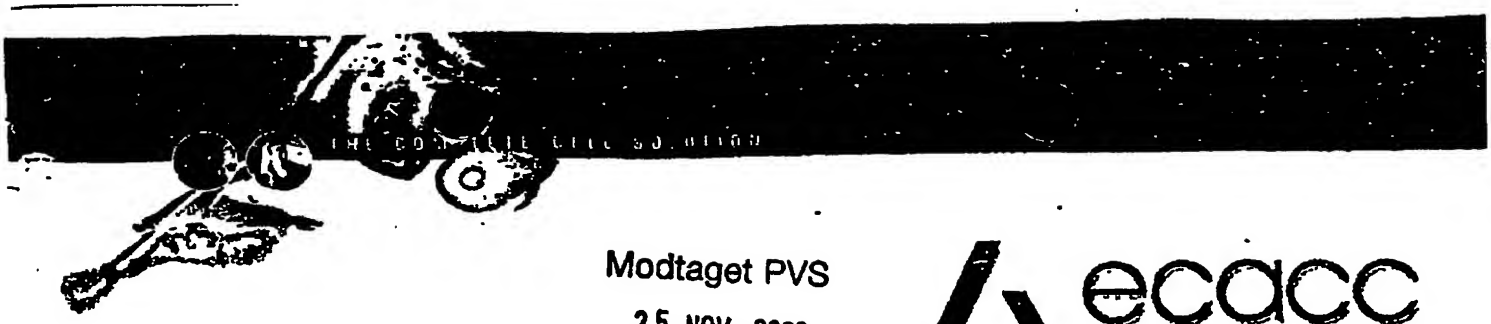
Please note that deposits which are, or contain, animal pathogens require an import licence into the EC. Please allow 8 weeks for this proc submit information requested by ECACC for licence applications as quickly as possible.

CAMR
Today's Research Tomorrow's Health

REGISTERED FIRE

No. FS33319

European Collection of Cell Cultures, Centre for Applied Microbiology & Research
Salisbury, Wiltshire SP4 0JG, UK.
Tel: +44 1980 612512 *Fax:* +44 1980 611315
E.Mail: ecacc@camr.org.uk *Web Site:* www.camr.org.uk



ecacc
European Collection of Cell Cultures

Centre for Applied Microbiology and Research
&
European Collection of Cell Cultures

This document certifies that Virus
(Deposit Ref. V00083008) has been accepted as a patent deposit,
in accordance with
The Budapest Treaty of 1977,
with the European Collection of Cell Cultures on 30TH August 2000

PS Packer
.....
Dr P J Packer
Quality Manager, ECACC

CAMR
European Collection of Cell Cultures, CAMR, Salisbury, Wiltshire SP4 0JG UK.
Tel: 44 (0) 1980 612512 Fax: 44 (0) 1980 611315 Email: ecacc@camr.org.uk Web Site: ecacc.org.uk

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]
V00083008 - MVA-BN VIABILITY OF MVA-BN WAS TESTED BY GROWING THE VIRUS ON BHK CELLS AND CALCULATING THE TCD50.

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Dr P J Packer ECACC CAMR Address: Porton Down Salisbury Wiltshire SP4 0JG	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 14/12/00 PSPade

4 Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

VIABILITY STATEMENT
Issued pursant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY OF STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: BAVARIAN NORDIC RESEARCH INSTITUTE GMBH Address: FRAUNHOFERSTRASSE 18B D-82152 MARTINSRIED GERMANY	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: V00083008 Date of the deposit or of the transfer: 30TH August 2000

II. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [2]. On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2 (a) (ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

BAVARIAN NORDIC RESEARCH
INSTITUTE GMBH
FRAUNHOFERSTRASSE 18B
D-82152 MARTINSRIED
GERMANY

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
MVA-BN	V00083008

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] A scientific description

[ ] A proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 30TH August 2000 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and
A request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

IV. INTERNATIONAL DEPOSITORY AUTHORITY

Name: Dr P J Packer

Address: ECACC
CAMR
Porton Down
Salisbury SP4 OJG

Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized officials(s):

Date: PJPacker 14/12/00

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired

Certificate of Analysis

Product Description MVA-BN
Accession Number 00083008

Test Description: The Detection of Mycoplasma by Isolation on Mycoplasma Pig Serum Agar and in Mycoplasma Horse Serum Broth.
SOP QC/MYCO/01/02

Acceptance Criterion/Specification: All positive controls (*M. pneumoniae & M. orale*) must show evidence of mycoplasma by typical colony formation on agar plates. Broths are subcultured onto Mycoplasma Pig Serum Agar where evidence of mycoplasma by typical colony formation is evaluated. All negative control agar plates must show no evidence of microbial growth.
The criteria for a positive test result is evidence of mycoplasma by typical colony formation on agar. A negative result will show no such evidence.

Test Number: 21487

Date: 27/11/00

Result:

Positive Control:	Positive
Negative Control:	Negative
Test Result:	Negative
Overall Result:	PASS

Test Description: Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system.
SOP QC/MYCO/07/05

Acceptance Criterion/Specification: The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence. Positive control (*M. orale*) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA. Positive test results appear as extra nuclear fluorescence of mycoplasma DNA. Negative results show no cytoplasmic fluorescence.

Test Number: 21487

Date: 27/11/00

Result:

Positive Control:	Positive
Negative Control:	Negative
Test Result:	Negative
Overall Result:	PASS

Authorised by.....PSPark.....**ECACC, Head of Quality**.....4/12/00.. **Date**

Certificate of Analysis

Product Description MVA-BN
Accession Number 00083008

Test Description: Detection of bacteria and fungi by isolation on Tryptone Soya Broth (TSB) and in Fluid Thioglycollate Medium (FTGM). SOP QC/BF/01/02

Acceptance Criterion/Specification: All positive controls (*Bacillis subtilus, Clostridium sporogenes* and *Candida albicans*) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear).
The criteria for a positive test is turbidity in any of the test broths. All broths should be clear for negative test result.

Test Number: 21487
Date: 27/11/00
Result:

Positive Control:	Positive
Negative Control:	Negative
Test Result:	Negative
Overall Result:	PASS

Test Description: Determination of $TCID_{50}$ of cytopathic Virus titration. (SOP ECACC/055) Cell

Acceptance Criterion/Specification/Criteria: Negative controls should show no sign of Cytopathic effects. The Test Sample is serially diluted into in 4 wells of indicator cell lines for each dilution. Cytopathic effects indicate that virus is present. Virus titre is calculated using the below equation where x is the value obtained from a standard $TCID_{50}$ Table as a result of the distribution of the wells displaying less than 4 positive wells per dilution, and y is the value of the highest dilution where all 4 wells are positive:

$$TCID_{50} = \frac{1}{y} \times 10^{1+x}$$

Date: 01/12/00

Result:

Indicator Cell Line:	BHK21 (Clone 13)
Negative Control:	NO CPE
Test Sample:	CPE
Distribution of less that 4 positive wells:	4, 4, 4, 3, 0
X:	1.25
Y:	10^{-7}

$$TCID_{50} = \frac{1}{10^{-7}} \times 10^{1+1.25}$$

$$= 10^{9.25}$$

Overall Result: Virus Present

*** End of Certificate ***

Authorised by.....RSA.....**ECACC, Head of Quality**.....4/12/00.... **Date**

ecacc European Collection of Cell Cultures

ECACC use only
Accession No:
Depositors Code:

Patent Deposit Accession Form - Virus

DEPOSITOR INFORMATION

Name of Depositor/Company/Institute: Bavarian Nordic Research Institute GmbH

(NB this will be the name that appears on certification)

Contact Name: Dr. Paul M. Howley, ~~Dr. Petra Pielken~~

Depositor Address: Fraunhoferstraße 18b, D-82152 Martinsried, Germany

Tel No: ++49 89 8565 0030 Fax No: ++49 89 8565 1333

BIOHAZARD STATEMENT MUST BE ENCLOSED

The deposit is made in accordance with the terms of the Budapest Treaty 1977. I agree to abide by the conditions and regulations regarding deposit of cell lines to the ECACC.

Signature: P. Pielken Date: 25.08.2000

Address to which invoice should be sent (if different from above)

Accounts Department, Bavarian Nordic Research Institute GmbH
Fraunhoferstraße 18b
D-82152 Martinsried, Germany

VIRUS INFORMATION

Name in full: Modified Vaccinia Virus Ankara

Abbreviated Name: MVA-BN Identification on Ampoules: Lot 010500

Strain: ~~Serological Type~~ vial #: 3; 32; 51; 76; 82; 85; 84; 88; 98; 99; 106; 109;

Normal Host: None

Virus Titre Deposited:

VIRUS PROPAGATION

Host cells (first choice): Chicken Embryo Fibroblast (CEF)

Alternative Host Cells: -

Details of Host Cell Growth (media, temperature, seeding density, growth factors etc)

Chicken Embryo Fibroblast Cultured in RPMI Media Supplemented with 10% FCS. AT 37°C/5%CO2. No Growth Factors Needed.

Details of Virus Growth (eg confluency of host cells, co-cultivation, moi, effects, time taken)

Infect CEF Cell At Near Cell Confluency (Approx. 90%) At MOI 0.1 TCID50/Cell

VIRUS STORAGE Confluency; Infection Times on Average 3 days At 37°C/5%CO2

Material stored (eg supernatant, infected cell extract, viable infected cells etc)

Temperature and conditions: Infected Cell Extract At-80°C

VIRUS ASSAY

Method (enclose if necessary)

Does not form Plaques. It forms Foci of CPE in CEF Monolayers. Titrate by TCID50 Method - Reference:

LITERATURE REFERENCES (if any) Ingo Drexler et al. 2000 in Methods in Molecular Medicine ~~Vol 35:~~ Gene Therapy: Methods and Protocol s Ed. W. and U. Stein. Human Press

ANY OTHER RELEVANT INFORMATION Virus Looses Viability At Low pH. Dilute Virus With Sterile IMM Tris-Hcl pH9 Buffer

European Collection of Cell Cultures, Centre for Applied Microbiology & Research
Salisbury, Wiltshire SP4 0JG, UK.
Tel: +44 1980 612512 *Fax:* +44 1980 611315
E.Mail: ecacc@camr.org.uk *Web Site:* www.camr.org.uk

CAMR Today's Research Tomorrow's Health

No. FS33819

ecacc
European Collection of Cell Cultures

ECACC use only
Accession No:
Depositors Code:

BIOHAZARD STATEMENT

(To be included with all deposits)

Deposit category

Cell Culture ☐ Plant Culture ☐ Virus Recombinant DNA ☐ DNA Probe ☐ Bacteria ☐

Does the above deposit represent an infectious, toxic or allergenic hazard? Yes ☐ No ☒

If yes, please give details and any associated hazard category (eg. ACDP category) and fax to ECACC PRIOR to shipment of cells.

Does the above deposit contain genetically manipulated material? Yes ☐ No ☒

If yes, please enclose a general description and answer the following:

a. is the material DNA ☐ RNA ☐

b. is the material present in a host organism? Yes ☐ No ☐

c. is the genetic material readily transferred to environmental organisms? Yes ☐ No ☐

d. is the genetic material likely to be expressed as protein? Yes ☐ No ☐

e. what is the category of this material under ACGM regulations?

ie, i. containment level _____

ii. GMO type _____

For any positive responses to questions b-d please give details

Please supply any further details which would be relevant to assessing the safe handling conditions for materials to be deposited at ECACC.

Highly attentuated Replication Incompetent in Humans and Animals

Signed P. Pielken Date 25.08.2000

Print name Dr. Petra Pielken

Please note that deposits which are, or contain, animal pathogens require an import licence into the EC. Please allow 8 weeks for this pro
submit information requested by ECACC for licence applications as quickly as possible.

CAMR
Today's Research
Tomorrow's Healt

European Collection of Cell Cultures, Centre for Applied Microbiology & Research
Salisbury, Wiltshire SP4 0JG, UK.
Tel: +44 1980 612512 *Fax:* +44 1980 611315
E.Mail: ecacc@camr.org.uk *Web Site:* www.camr.org.uk

PHLS Public Health Laboratory Service

Centre for Applied Microbiology and Research

This document certifies that Virus Strain
(Deposit ref V94012707 *) has been accepted*
as a patent deposit, in accordance with
The Budapest Treaty of 1977,
with the European Collection of Animal Cell Cultures on
27th January 1994

Dr. Alan Doyle,
Curator.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Prof Dr Dr h.c. mult
Anton Mayr
Bockmeyrstrasse 9
80992 Munchen
Germany
NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:
Vacciniavirus Strain MVA

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
V94012707

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 27/1/94 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Dr A. Doyle

Address: ECACC, CAMR
Porton Down
Salisbury, SP4 OJG, UK

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 28th June 1994

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Prof Dr Dr h.c. mult Anton Mayr
Bockmeyrstrasse 9
80992 Munchen
Germany

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified on the following page

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Prof Dr Dr h.c. mult Anton Mayr Address: Bockmeyrstrasse 9 80992 Munchen Germany	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: V94012707 Date of the deposit or of the transfer: 27th January 1994

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 27th January 1994 [2]. On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Dr A. Doyle Address: ECACC CAMR Porton Down Salisbury, SP4 OJG, UK.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 28th June 1994

[4] Fill in if the information has been requested and if the results of the test were negative.